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(54) Title: POLY(ORGANO)PHOSPHAZENES FOR USE IN SYNTHETIC TRANSFECTION SYSTEMS

(57) Abstract

The present invention relates to a synthetic transfection system comprising as a carrier a cationic, water soluble or water dispersible polyphosphazene. In addition, it relates to a method for introducing DNA fragments in target cells, comprising contacting these DNA fragments with a polyphosphazene which is at least partially substituted with cationic substituents and subsequently contacting the obtained transfection system wits target cells. Finally, the invention involves the use of a polyphosphazene which is at least partially substituted with cationic substituents as transfection vehicle.

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Title: Poly(organo)phosphazenes for use in synthetic transfection systems

The present invention relates to the field of synthetic transfection systems useful in the delivery of gene constructs or DNA fragments to cells, especially to cells in living organisms. More in particular, the invention relates to cationic polymers having broad possibilities to be modified or adapted in order to create a flexible DNA gene delivery system, which can be used in, e.g., gene therapy applications.

Gene therapy is seen as a promising method to correct hereditary defects or to treat life threatening diseases such as cancer and AIDS. In gene therapy, nucleic acid fragments or gene constructs are brought into target cells. These nucleic acid fragments or gene constructs are preferably incorporated in plasmids or other vectors.

If the reconstructed plasmids are applied to an organism per se, this generally leads to low expression of the introduced gene, if any. There are three main reasons for this low expression. First, the plasmids will hardly ever reach the cell population where they are intended to be incorporated, due to degradation and elimination processes. Second, if the plasmids do reach the target cells, they cannot simply pass the cellular membrane, because of the strongly polar nature and the size of the plasmids. Third, if a plasmid does invade a target cell, it normally will be enclosed in an endosome, which will convert into a lysosome. In the lysosome, the plasmid will be degraded so that the incorporated gene cannot be expressed.

For the above reasons, in gene therapy plasmids comprising a desired gene construct are transported to and delivered in the target cells by means of carrier systems.

In recent years, many efforts have been made in the research on potentially suitable transfection systems, both of viral and non-viral origin. These transfection systems should deliver the desired gene to the target cell and cause it to be expressed to a high degree.

Viral vectors are very suitable, because by nature adapted, to introduce plasmids in target cells and to avoid endosome disruption, the degradation of the plasmids in endosomes or the transition of endosomes into lysosomes. However, viral vectors have a number of pronounced disadvantages. Viral vectors are able to effect integration of the introduced gene in the chromosomal DNA of the target cell. The site where this integration is effected cannot (yet) be predicted or controlled, which involves the risk of destroying essential genes or activation of, e.g., oncogenes. In addition, it is at present very difficult to provide for viral vectors on a commercial scale. Moreover, viral vectors generally trigger the immune system of a living organism, which will lead to immuno responses against the transfection system when used *in vivo*. Finally, viral vectors inherently set limits to the size of the gene construct to be introduced in the target cell.

In order to overcome the intrinsic disadvantages of viral vectors, synthetic transfection systems should offer good perspectives.

In this light, a review article of Kabanov et al. in Bioconjugate Chemistry vol.6, no. 1 (1995), 7-20 is mentioned. This review article describes in general terms the principle of delivery of genetic material onto cells employing soluble in terpolyelectrolyte complexes (IPEC's) of nucleic acids with linear polycations. It is indicated that various polycations have been used to produce IPEC's. As explicit examples polyvinyl pyrimidinium salts, polypeptides such as polylysine conjugates and lipopolylysines, and spermines are mentioned.

Explicit reference is made to research carried out by the group of E. Wagner, relating to gene delivery by means of plasmid-polylysine complexes (Curiel et al., Adenovirus Enhancement of Transferrin-Polylysine-Mediated Gene Delivery, Proc. Natl. Acad. Sci. 88 (1991) 8850-8854; Plank et al., Gene Transfer into Hepatocytes Using Asialloglycoprotein Receptor Mediated Endocytosis of DNA Complexed with an Artificial Tetra-Antennary Galactose Ligand Bioconj. Chem. 3 (1992) 533-

539; Wagner et al., Influenza Virus Hemagglutin HA2 N-Terminal Fusogenic Peptides Augment Gene Transfer by Transferrin-Polylysine-DNA Compleses: Toward a Synthetic Virus-like Gene-Transfer Vehicle, Proc. Natl. Acad. Sci. 89 (1992) 7934-7938; and Curiel et al., Gene Transfer to Respiratory Epithelial Cells via the Receptor Mediated Endocytosis Pathway, Am. J. Respir. Cell Mol. Biol. 6 (1992) 247-252). The plasmid-polylysine complex investigated upon exposition to certain cell lines showed at least some expression of the gene. Further, it was found that the expression efficiency increased considerably due to the binding of transferrin to the plasmid-polylysine complex. Transferrin gives rise to close cell-complex contact; it binds the entire complex to the transferrin receptor of cells. Subsequently, at least part of the entire complex was found to be incorporated in the cell.

However, the transfection efficiency of these polylysine based transfection systems as well as other known synthetic transfection systems is much lower than the efficiency of the known viral vectors.

The aim of the present invention is to provide for an effective and efficient synthetic transfection or blocking system. Such a system should fulfil the following conditions. The synthetic carrier system used must be biocompatible and preferably biodegradable. In order to be able to bind and condense DNA, e.g. in the form of a plasmid, wherein a gene construct is incorporated, the carrier system should possess a positive charge at physiological pH.

It has now been found that such a system can be based on polyphosphazenes which are at least partially substituted with cationic substituents. More in particular, the invention relates to water soluble or water dispersible poly(organo)-phosphazene based transfection systems, wherein organic cationic moieties are attached to the polyphosphazene backbone.

The polyphosphazene polymer which forms the basis of the poly(organo)phosphazene systems of the present invention essentially comprises a backbone of $-[-P(R)_2=N-]_n-$ units,

wherein $(R)_2$ represents two groups, which may or may not be the same, coupled to the phosphorous atom. A restricted number of other units may, however, be present.

It is already known from other technical fields that polyphosphazenes are biocompatible and biodegradable. Polyphosphazenes were studied in the art of biomedical and pharmaceutical applications. In this light, reference is made to, e.g., the PhD Thesis of J. Goedemoed titled "Polyphosphazene Drug Delivery Systems for Antitumor Treatment", University of Leiden (1990); to Crommen et al. Biodegradable Polymers I, Synthesis of Hydrolysis-Sensitive Poly(organo)phosphazenes, Biomaterials 13 (1992), 511-520; to Domb et al. in Polymer Advanced Technology vol. 3, no. 6 (1992) 279-292; to Calicetti et al. in Il Farmaco vol. 49, no. 1, (1994) 69-74; and Andrianov et al. in J. Control Release vol. 27, no. 1 (1983) 69-77. These other technical fields essentially relate to controlled and sustained release systems.

In order to be able to bind to and condense with plasmids, the poly(organo)phosphazene used must - at physiological pH - at least contain 5% cationic radicals or groups coupled to the phosphorous atom in the $-[-P(R)_2=N-]_n-$ units. Preferably, at least 40% and more preferably 50% cationic groups are coupled to the phosphorous atoms. In these cases, the polyphosphazene is able to bind DNA electrostatically and condense therewith. Moreover, degradation and elimination of the DNA in the systemic environment is avoided. In addition, it appeared that such polyphosphazene-DNA complexes are taken up in the target cells in a considerably higher amount as compared with the plasmids per se.

Suitable cationic substituents are preferably derived from organic moieties possessing an amino group. Such moieties are positively charged at physiological pH. Examples of these organic moieties are amino C_{1-10} alcohols, and amino C_{1-10} alkoxy C_{1-10} alcohols, as well as their secondary, tertiary and

quaternary derivatives. Especially, tertiary amines are preferred.

Although it is important that the polymer is positively charged as a whole, it is essential that the phosporous atoms in the poly phosphazene backbone not entirely comprise cationic moieties. Polyphosphazenes which are entirely substituted with cationic substituents essentially do not give rise to expression of the transported gene constructs. More in particular, part of the phosphorous atoms, preferably at least 10%, should be coupled to other hydrophobic and hydrophilic moieties.

Especially good results are obtained when at least 10% of the substituents coupled to the phosphorous atoms in the polymer backbone are derived from essentially electrically neutral organic hydrophilic substituents such as glycerol, methoxy ethoxy ethanol and polyethylene glycol. A possible explanation for these advantageous results is that the plasmid will be less tightly bound to the polymer, so that it can dissociate easier in the target cell. Further, it is noted that PEG avoids recognition by macrophages.

Hydrophobic groups, such as benzyl alcohol and lauryl alcohol, probably assist in transfer of the membranes of target cells.

The polyphosphazene used in accordance with the present invention is of a cationic nature, and it is water soluble or water dispersible. More in particular, very good results are obtained if one of the substituents R in the formula $-[-P(R)_2 = N-]_n-$ is a cationic group while the other substituent R may represent in 5-100% of the occurrences a cationic moiety and in 95-0% of the occurences an anionic or neutral moiety.

The molecular weight of the polyphosphazene polymers used can be easily adjusted to the size of the plasmid to be transported. Normally, polyphosphazenes having a molecular weight of from 10,000 to 1,000,000 can suitably be used. The weight ratio of the polyphosphazene polymers to the DNA fragments is very critical. The transfection efficiency does

increase with increasing polymer to plasmid ratios. Suitable results are obtained when using weight ratios of between 0.1 and 200, preferably this ratio is higher than 1 most preferably higher than 7. The length of the polyphosphazene polymers can be controlled by using and maintaining suitable reaction conditions in the polymerization process.

The synthetic transfection system of the invention which comprises at least one cationic, water soluble or water dispersible polyphosphazene as a carrier further comprises a DNA fragment such as a vector, a plasmid, a gene construct or an oligonucleotide. Oligonucleotides can be used as a blocking structure in cells.

It is noted that the condensed particles comprising the polyphosphazene and DNA fragments can be enclosed or incorporated in known drug delivery systems, e.g. in liposomes.

Genes to be incorporated into vectors or vehicles to be used in the synthetic transfection system are among others documented in

- McKusick, V.A. Mendelian inheritance in man, catalogs of autosomal dominant, autosomal recessive, and X-linked phenotypes. Eighth edition. John Hopkins University Press (1988).
- Stanbury, J.B., Wyngaarden, J.B., Frederickson, D.S., Goldstein, J.L. and Brown, M.S. The metabolic basis of inherited disease. Fifth edition. McGraw-Hill (1983).

These genes include but are not limited to :
genes associated with diseases of the carbohydrate metabolism such as for:

- fructose-1-phosphate aldolase
- fructose-1,6-diphosphatase
- glucose-6-phosphatase
- lysosomal α-1,4-glucosidase
- amylo-1,6-glucosidase
- amylo-(1,4:1,6)-transglucosidase
- muscular phosphorylase
- liver phosphorylase
- muscular phosphofructokinase

- phosphorylase-b-kinase
- galactose-1-phosphate uridyl transferase
- galactokinase
- all enzymes of the pyruvate dehydrogenase complex
- pyruvate carboxylase
- 2-oxoglutarate glyoxylate carboligase
- D-glycerate dehydrogenase;

genes associated with diseases of the amino acid metabolism such as for:

- phenylalanine hydroxylase
- dihydrobiopterin synthetase
- tyrosine aminotransferase
- tyrosinase
- histidase
- fumarylacetoacetase
- glutathione synthetase
- γ-glutamylcysteine synthetase
- ornithine-δ-aminotransferase
- carbamoylphosphate synthetase
- ornithine carbamyltransferase
- argininosuccinate synthetase
- argininosuccinate lyase
- arginase
- L-lysine dehydrogenase
- L-lysine ketoglutarate reductase
- valine transaminase
- leucine isoleucine transaminase
- "branched chain" 2-keto acid decarboxylase
- isovaleryl CoA dehydrogenase
- acyl-CoA dehydrogenase
- 3-hydroxy-3-methylglutaryl CoA lyase
- acetoacetyl CoA 3-ketothiolase
- propionyl CoA carboxylase
- methylmalonyl CoA mutase
- ATP:cobalamine adenosyltransferase
- dihydrofolate reductase

- methylene tetrahydrofolate reductase
- cystathionine β-synthase
- sarcosine dehydrogenase complex
- proteins belonging to the glycine cleavage system
- β-alanine transaminase
- serum carnosinase
- cerebral homocarnosinase;

genes associated with diseases of fat and fatty acid metabolisms such as for:

- lipoprotein lipase
- apolipoprotein C-II
- apolipoprotein E
- other apolipoproteins
- lecithin cholesterol acyltransferase
- LDL receptor
- liver sterol hydroxylase
- "Phytanic acid" α-hydroxylase;

genes associated with lysosomal defects such as for:

- lysosomal α-L-iduronidase
- lysosomal iduronate sulphatase
- lysosomal heparan N-sulphatase
- lysosomal N-acetyl-α-D-glucosaminidase
- lysosomal acetyl CoA:α-glucosaminide N-acetyltransferase
- lysosomal N-acetyl-α-D-glucosaminide 6-sulphatase
- lysosomal galactosamine 6-sulphate sulphatase
- lysosomal β-galactosidase
- lysosomal arylsulphatase B
- lysosomal β-glucuronidase
- N-acetylglucosaminylphosphotransferase
- lysosomal α-D-mannosidase
- lysosomal α-neuraminidase
- lysosomal aspartylglycosaminidase
- lysosomal α-L-fucosidase
- lysosomal acid lipase
- lysosomal acid ceramidase

- lysosomal sphingomyelinase
- lysosomal glucocerebrosidase
- lysosomal galactosylceramidase
- lysosomal arylsulphatase A
- α-galactosidase A
- lysosomal acid β-galactosidase
- α-chain of the lysosomal hexosaminidase A;

genes associated with diseases of the steroid metabolism such as for:

- 21-hydroxylase
- 11β-hydroxylase
- androgen receptor
- steroid 5α-reductase
- steroid sulphatase;

genes associated with diseases of the purine and pyrimidine metabolism such as for:

- phosphoribosylpyrophosphate synthetase
- hypoxanthine guanine phosphoribosyltransferase
- adenine phosphoribosyltransferase
- adenosine deaminase
- purine nucleoside phosphorylase
- AMP deaminase
- xanthine oxidase
- orotate phosphoribosyltransferase
- orotidine 5'-phosphate decarboxylase
- DNA repair enzymes;

genes associated with diseases of the porphirine and haemal metabolism such as for:

- uroporphyrinogene III cosynthase
- ferrochelatase
- porphobilinogene deaminase
- coproporphyrinogene oxidase
- proporphyrinogene oxidase
- uroporphyrinogene III synthase

- uroporphyrinogene decarboxylase
- bilirubine UDP-glucuronyltransferase
- catalase;

genes associated with diseases of the connective tissue, muscles and bone such as for:

- lysyl hydroxylase
- procollagen peptidase
- α1-antitrypsine
- dystrophine
- alkaline phosphatase
- guanosine nucleotide regulatory protein of the adenyl cyclase complex;

genes associated with diseases of the blood and blood-forming organs such as for:

- blood coagulation factor V
- blood coagulation factor VII
- blood coagulation factor VIII
- blood coagulation factor IX
- blood coagulation factor X
- blood coagulation factor XII
- blood coagulation factor XIII
- all other blood coagulation factors
- all genes associated with osteopetrosis such as for: "carbonic anhydrase II"
- thrombocytes membrane glycoprotein Ib
- thrombocytes membrane glycoprotein IIb-IIIa
- spectrin
- pyruvate kinase
- glucose-6-phosphate dehydrogenase
- NADH cytochrome b_5 reductase
- β-globin
- α-globin;

genes associated with diseases of transport systems such as for:

- lactase
- sucrase-α-dextrinase
- 25-hydroxyvitamin D_3-1-hydroxylase
- cystic fibrosis transmembrane conductance regulator (CFTR);

genes associated with congenital immunodeficiencies such as for:

- the proteins of the complement system including B, C1q, C1r, C2, C3, C4, C5, C7, C8 and C10
- the inhibitor of C1, a component of the complement system
- the inactivator of C3b, a component of the complement system;

the genes for X-bound immunodeficiencies such as for:

- one of the enzymes of the NADPH oxidase complex
- myeloperoxidase
- the syndrome of Wiscott Aldrich and Ataxia Telangiectasia;

genes coding for hormones as well as the genes coding for their receptors such as for instance for:

- growth hormone.

Further, these genes also include genes which (to date) have not been associated with a hereditary defect but with which gene therapy can be practised in some manner.

These include:

the gene for tyrosine hydroxylase;

drug resistance genes such as for instance:

- the P-glycoprotein P170 (the so-called multi drug resistance gene mdr1)
- mdr 3
- dihydrofolate reductase (DHFR) and methotrexate resistant isotypes thereof

- metallothioneine
- aldehyde dehydrogenase (ALDH)
- glutathione transferase;

genes coding for all cytokins including for instance all interleukins and all interferons;

genes coding for all growth factors;

genes coding for all growth factor receptors;

genes coding for all transplantation antigens such as for instance the major and minor histocompatibility antigens;

genes capable of affording resistance against infectious organisms, such as for instance TAR decoys;

genes of infectious organisms which can be used for vaccination purposes such as for instance the envelope gene of HIV;

genes which can be used for negative selection such as for instance the thymidine kinase gene of the Herpes simplex virus against which selection can be effected with substrates such as for instance gancyclovir or acyclovir.

Vectors to be used include viral and non-viral regulatory elements for expression and/or replication. These vectors are well known in the field.

Suitable transfection systems are able to target a gene construct to the aimed cell population. The polyphosphazene based transfection system of the invention therefore at least comprises one group that is selectively targetted to target molecules associated with the surfuace of the target cells. Such targeting moieties or homing devices are known to the skilled person and comprise, e.g., tri and tetra antennary cluster glycosides, transferrin or other protein contructs, monoclonal antibodies against cell membrane proteins, ligands

for cell surface associated receptors and binding fragments of derivatives of said targeting moieties etc.. If, for instance, galactose moieties are coupled to the polyphosphazene system of the present invention the transported gene fragments are incorporated in hepatocytes through the galactose receptor of hepatocytes. Furthermore, the presence of recognizable structures covalently or non-covalently coupled to the polyphosphazene part of a polyphosphazene-DNA complex facilitates the incorporation of the gene construct in the target cell.

Moreover, the transfection system can be adapted to allow the gene construct to leave endosomes in the cellular system. Thereto membrane destabilizing structures, in particular polypeptide fragments, are conjugated to the poly(organo)phosphazene systems. Such destabilizing structures should be able to disturb or destabilize the endosomal membrane systems. The plasmids incorporating a gene construct so reach the cytoplasm of the target cell, where the gene construct can be expressed. Examples of such membrane destabilizing structures which are suitably used in accordance with the present invention are fusogenic structures, e.g. certain peptides and (parts of) viral coating proteins, for instance peptides derived from hemagglutinin protein of the influenza virus (see in this respect, e.g., Plank et al. The Influence of Endosome-Disruptive Peptides on Gene Transfer Using Synthetic Virus-Like Gene Transfer Systems, J. Biol. Chem. 269 (1994), 12918-12924).

Another compound that is useful in accordance with the present invention is chloroquine. It is noted that chloroquine is only used in *in vitro* applications, because it is toxic *in vivo*. Since the invention is directed to both *in vivo* and *in vitro* applications, this embodiment is within the scope of the invention.

As said herein above polyphosphazenes are known per se. This also applies to methods of preparing these polymers. A preferred method to prepare polyphosphazenes starts from hexachlorotriphosphazene. This starting compound is subjected

to ring opening polymerization yielding poly dichlorophosphazene. Ring opening polymerization of hexachloro triphosphazene in the bulk is for instance described in Kircheldorf (ed.) Handbook of Polymer Synthesis Part B, Chapter 17, Phosphorous-Containing Polymers (1991); while solution polymerization is described in Mujumdar et al. Solution Polymerization of Selected Polyphosphazenes, Macromol. Chem. 190, (1989) 2293-2302.

Poly(organo)phosphazenes used in accordance with the present invention can be prepared from these poly dichloro phosphazenes by effecting nucleophilic substitution with organic compounds which are cationic at physiological pH or can easily be converted to such cationic substituents.

Random copolymers can be synthesized by carrying out the substitution reaction in the simultaneous presence of two or more substituents. It is also possible to subject the starting polymer first to a reaction with a first substituent and subsequently with another substituent. Suitable methods of these nucleophilic substitutions are well within the scope of the person skilled in the art.

Fusogenic peptides and targeting molecules can be bonded to the poly(organo)phosphazenes using well-known techniques, for instance using thiol groups introduced in the polymer and maleimide groups introduced in the peptide or targeting molecule.

In a preferred embodiment, the polyphosphazene polymer is substituted with N,N-dimethylamino ethanol groups. These cationic groups are introduced by contacting polydichloro-phosphazenes with N,N-dimethylamino ethanol.

In a further embodiment, the invention relates to a method for introducing DNA fragments in target cells, comprising contacting these DNA fragments with a polyphosphazene which is at least partially substituted with cationic substituents and subsequently contacting the obtained transfection system with target cells.

Finally, the invention relates to the use of a polyphosphazene which is at least partially substituted with cationic substituents as transfection vehicle.

The polyphosphazene system can be used both in *in vivo* and in *in vitro* applications.

In the figures

Fig. 1 shows the number of transfected COS-7 cells and the relative cell viability dependent on the polymer/plasmid ratio;

Figs. 2-3 show the number of transfected OVCAR cells and the relative cell viability dependent on the polymer/plasmid ratio;

Fig. 4 shows the percentage of protonated amine side chains as function of the pH;

Fig. 5 shows the degradation of N,N-dimethyl-aminoalcohol substituted polyphosphazene (DS 75%) at 37°C, pH 7.2; and

Fig. 6 shows the number of transfected OVCAR cells and the relative cell viability as a function of the polymer/plasmid ratio.

The present invention will be described in further detail while referring to the following examples.

Example 1

Water-soluble poly(organophosphazenes) with cationic side groups were synthesized as follows. Cyclic phosphazene trimer (hexachlorotriphosphazene) was polymerized to poly(dichloro)phosphazene by a solution polymerization using the method described by Mujumdar et al.(Macromolecules 1990 23, 14-21).

In a round bottom flask equipped with a condensor, hexachlorotriphosphaze (12 g; Aldrich, Belgium) was dissolved in 1,2,4-trichlorobenzene (10 ml, Aldrich, Belgium). Catalyst (sulfamic acid, 50 mg) and promotor ($CaSO_4.2H_2O$, 25 mg) were added. The reaction mixture was heated to 214°C while stirring using a magnetic stirrer. The reaction was carried out under a nitrogen atmosphere. After a reaction time of 1 hour and

25 minutes, the reaction mixture was cooled to room temperature. Anhydrous hexane (20 ml) was added to precipitate the polymer and to extract unreacted monomer and low molecular weight products. The hexane phase was removed and the polymer was dissolved in about 50 ml anhydrous THF.

In a separate reaction, the sodium salt of N,N-dimethylethanolamine was prepared. To a solution of N,N-dimethylethanolamine (17 ml) in anhydrous THF (250 ml) in a three-neck round bottom flask equipped with a condensor. Na spheres (diameter 3-8 mm, 5 g) were added. This mixture was refluxed four 24 hours. After cooling to room temperature an additional 500 ml of THF was added, followed by the addition of the solution of poly(dichlorophosphazene) in THF (see above). After a reaction time of 7 days at room temperature, THF was evaporated under reduced pressure. The polymer was dissolved in about 250 ml water and extensively dialyzed against RO (reverse osmosis) water. The polymer was collected by lyophilization (yield 1-2 g). The degree of substitution was established by ^{1}H-NMR, ^{31}P NMR and titration experiments and amounted to 60%. It is assumed that the unsubstituted P-Cl groups (still present in the polymer after the reaction of poly(dichlorophosphazene) with the sodium salt of dimethylethanolamine) are converted into POH groups after contact with water. The weight average molecular weight (Mw) and number average molecular weight (Mn) relative to dextran were 52.000 g/mol and 19.000 g/mol respectively (GPC analysis, eluens: 0.8 M $NaNO_3$ in water).

A simular rection wherein the poly(dichlorophosphazene) was reacted under refluxing with the sodium salt of dimethylethanolamine for 7 days resulted in a polyer with a Mw = 26.000 g/mol and Mn = 12.500 g/mol; the degree of substitution amounted to 75%.

The transfection efficiency of polymer/plasmid complexes was evaluated in COS-7 and/or OVCAR 3-cells according to the protocol as described by J.Y. Cherng et al. (Pharm. Res. In press), PCMV-lacZ plasmid (7.8 kB) containing a bacterial lacZ gene preceded by a Nuclear Location Signal under control of

the CMV promoter (A. Bout et al. Exp. Lung Res.19 193-202, (1993) was used as reported gene. The polymer/plasmid complexes were prepared by adding 400 µl of cationic polyphosphazene (varying concentration too 100 µl of plasmid (final concentration 5 µg/ml). Both the plasmid and the polymer were dissolved in RPMI. This mixture was incubated for 1 hour at room temperature. The number of transfected cells was established by counting the number of blue spots in a well. The relative cell viability was determined using the XTT assay (DA Scudiero et al., Cancer Res. 48, 4827-4833, 1988). Figure 1-3 shows the results.

Dynamic light scattering experiments showed that at a polymer/plasmid ratio > 15 (w/w), particles with a hydrodynamic diameter of 200-300 nm were formed. At lower polymer/plasmid ratios, substantially larger particles (1000 nm) were formed. The polymer used had a Mw of 52.000 g/mol and a degree of substitution of 60%.

The degree of protonation under physiological conditions (pH 7.2, 0.9% NaCl) was determined by potentiometric titration. Figure 4 shows the results. from this figure is appears that at pH 7.2 about 65% of the amine side chains is protonated.

The degradation of polymer (Mw = 26.000 g/mol, Mn = 12.500) degree of substitution 75%) was studied. Therefore a solution of this polymer (26.1 mg) in 10 ml Hepes buffer (5 mM, pH 7.2) also containing 0.02% NaN_3 was incubated at 37°C. Periodically samples were withdrawn and analyzed by gel permeation chromatography (eluens 0.8 M $NaNO_3$). Molecular weights were determined relative to dextran. The results are shown in Figure 5.

Example 2

Poly(dichlorophosphazene) was synthesized essentially as described under example 1. Part of the Cl groups was substituted with N,N-dimethylethanolamine and part with PEGME (poly(ethyleneglycol)methylether), M = 550 g/mol; Aldrich). This was carried out by refluxing a solution of poly(dichloro-

phosphazene) and a mixture of the sodium salt of N,N-dimethylethanolamine (51 ml) and PEGME (40 ml) in THF (800 ml) for 4 days. The polymer was collected after dialysis and lyophilization as described under example 1. The molar ratio of N,N-dimethylethanolamine and PEGME as determined by ^{1}H-NMR amounted to 2.Mw = 600.000 g/mol, Mn = 54.000 g/mol. The transfection efficiency of polymer/plasmid complexes was evaluated in OVCAR-3 cells (see example 1). The results are shown in Figure 6.

The transfection efficiency of polymer/plasmid was also evaluated in 911 cells, described by Fallaux et al., Hum. Gene Ther. 7, 215-222, 1996.

The plasmid used for transfection was pCMV.lacZ (Bout et al., Exp. Lung. Res. 19, 193-202, 1993).

911 cells were cultured in Dulbecco's Modified Essential Medium (DMEM) in the presence of 10% Fetal Bovine Serum (FBS). On day 0, 10^6 911 cells were seeded in 24 well plates (Nunc). On day 1, the culture medium was aspirated and DMEM (without FBS) containing different amounts of polymer/plasmid DNA was added to the cells in duplicate, in a volume of 500 µl. The ratios of polymer/plasmid (w/w) is indicated in the Table below. Per well of the 24 wells plate, 0.83 µg of plasmid DNA was added. 1 hour after addition of the DMEM/polymer-plasmid to the cells, 500 µl of (DMEM + 10% FBS) was added to the cells. After overnight incubation, the medium was replaced by 1 ml of DMEM + 10% FBS. The cells were incubated for another 24 hours and then fixed and stained with X-GAL, as described in example 1. The percentage of X-GAL positive cells, recognizable by blue nuclei, was determined.

The results were as follows:

co-polymer/plasmid ratio (w/w)	% blue cells
28:1	23
14:1	9
7:1	0.5
3:1	<0.5

homo-polymer/plasmid ratio (w/w)	% blue cells
28:1	20
14:1	7
7:1	3
3:1	0.5

The results indicate that a significant fraction of culture 911 cells can be transfected using poly-organophosphazenes. The transfection efficiency on 911 cells is increasing with increasing ratios of polymer to plasmid.

Example 3

Poly(dichlorophosphazane) with varying molecular weights was synthesized essentially as described under example 1.

In a round bottom flask equipped with a condensor, hexachlorotriphosphaze (6 g) was dissolved in 1,2,4-trichlorbenzene (5 ml). Next, a varying amount of catalyst (sulfamic acid: 25, 100 a=or 350 mg) and a fixed amount of promoter ($CaSO_4.2H_2O$, 25 mg) were added. The reaction mixture was heated to 214°C under constant stirring using a magnetic stirrer in an inert atmosphere (dry nitrogen) for 75, 65 and 41 minutes respectively. The resulting polymers were precipitated in hexane, dissolved in anhydrous dioxane and subsequently added to a solution of the sodium salt of N,N-dimethylethanolamine (19 ml and 5 g Na spheres) in dioxane (600 ml). This reaction mixture was refluxed for 24 hours until no P-Cl groups (IR-analysis, 515 cm-1) could be detected. Thereafter a solution of the sodium salt of 2,2,2-trifluorethanol (3.2 ml and 1 g Na spheres) in dioxane (250 ml) was added to quench any residual P-Cl groups.

The polymers were isolated essentially as described under example 1. The next table gives the molecular weights (GPC analysis, relative to dextran).

mg catalysis	MW (g/mol)	Mn (g/mol)
25	30500	11000
100	23600	9200
350	9700	4500

CLAIMS

1. A synthetic transfection system comprising as a carrier a cationic, water soluble or water dispersible polyphosphazene.

2. The system according to claim 1, further comprising a plasmid, a vector, a gene construct or an oligonucleotide.

3. The system of claim 1 or 2, wherein at least 5% of the groups bonded to the backbone phosphorous atoms of the polyphosphazene are cationic at physiological pH.

4. The system of any one of claims 1-3, wherein condensed particles comprising the polyphosphazene and DNA fragments are enclosed in drug delivery systems such as liposomes.

5. The system of any one of the preceding claims, wherein the polyphosphazene polymer is coupled to a homing device.

6. The system of any one of the preceding claims, wherein the polyphosphazene polymer is coupled to galactose or transferrin.

7. The system of any one of the preceding claims, wherein the polyphosphazene polymer is coupled to a fusogenic structure.

8. A method for introducing DNA fragments in target cells, comprising contacting these DNA fragments with a polyphosphazene which is at least partially substituted with cationic substituents and subsequently contacting the obtained transfection system with target cells.

9. Use of a polyphosphazene which is at least partially substituted with catonic substituents as transfection vehicle.

10. In vivo use of claim 9.

Figure 1:

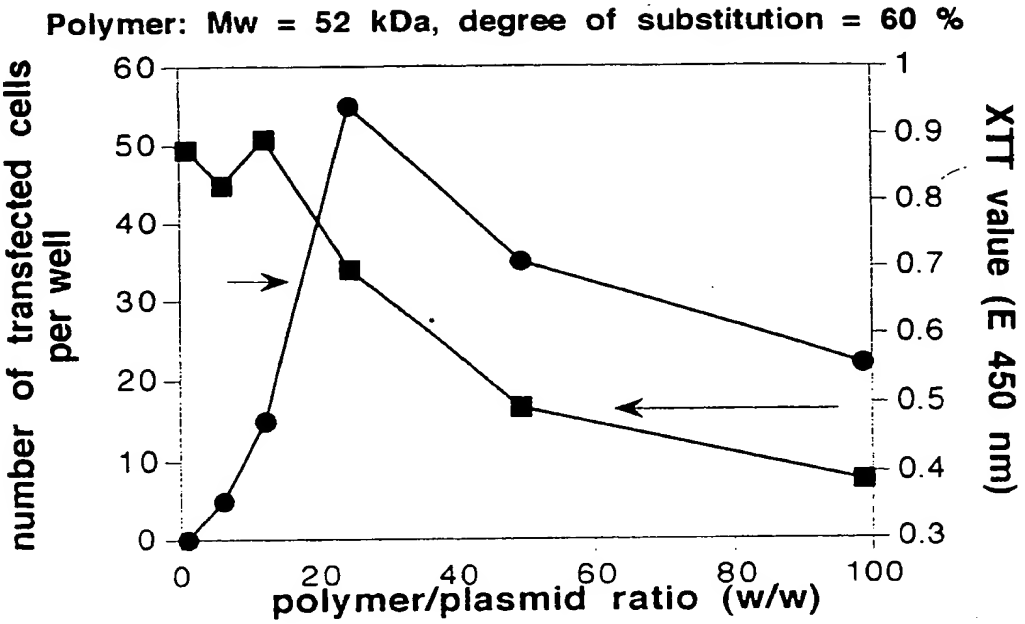
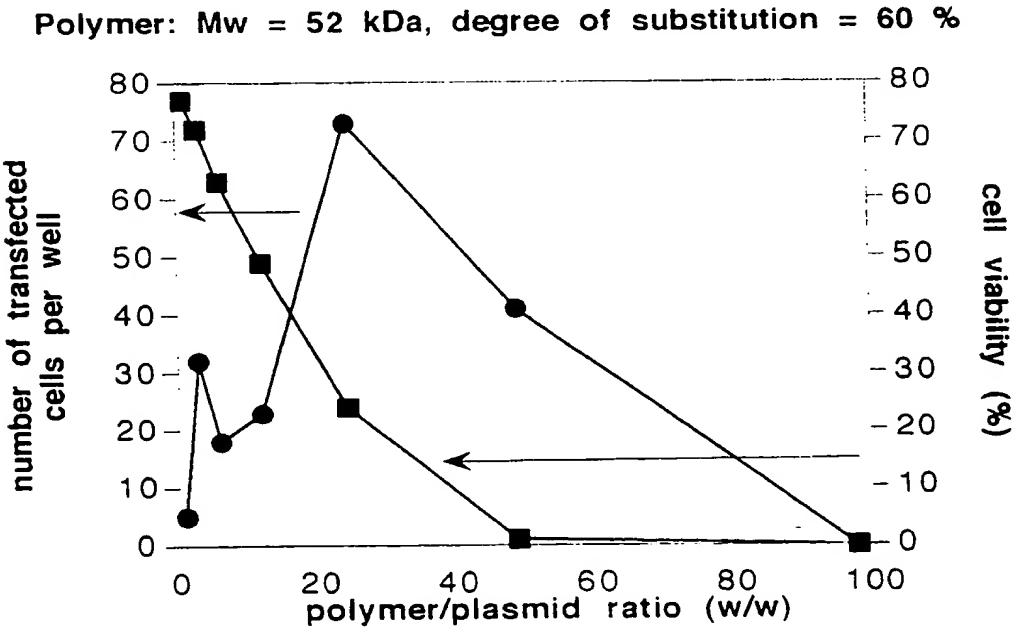


Figure 2:



Figur 3:

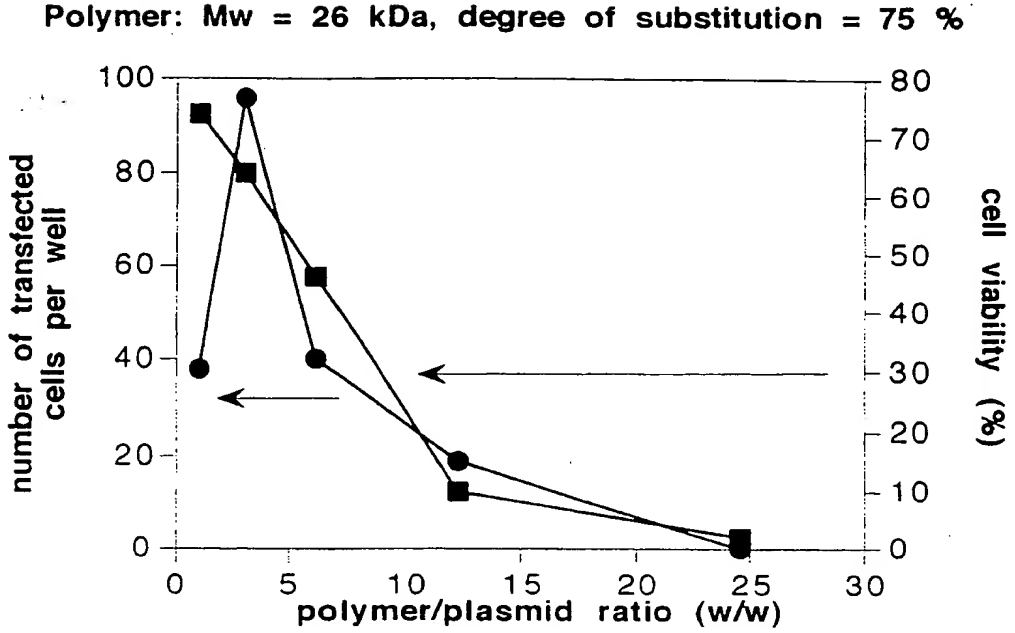


Figure 4:

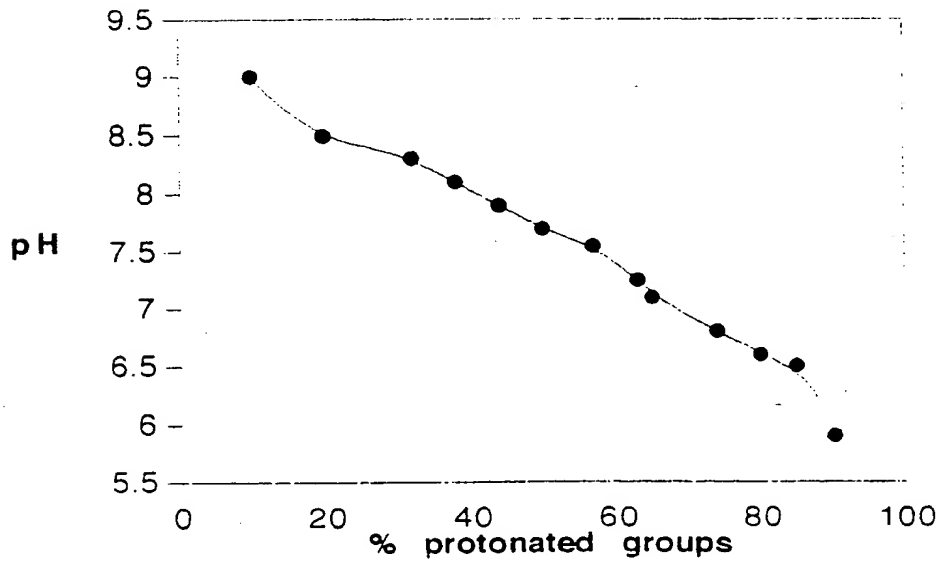


Figure 5:

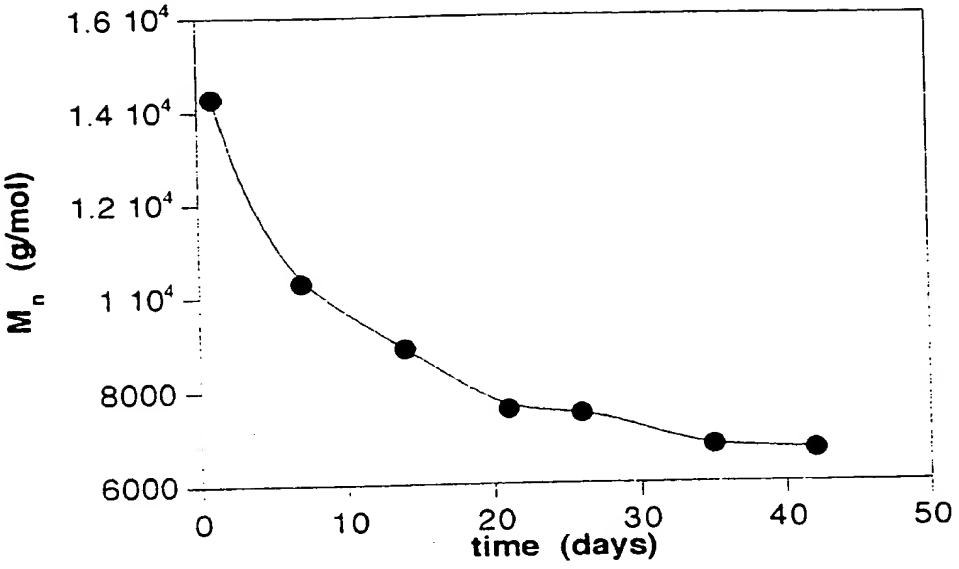
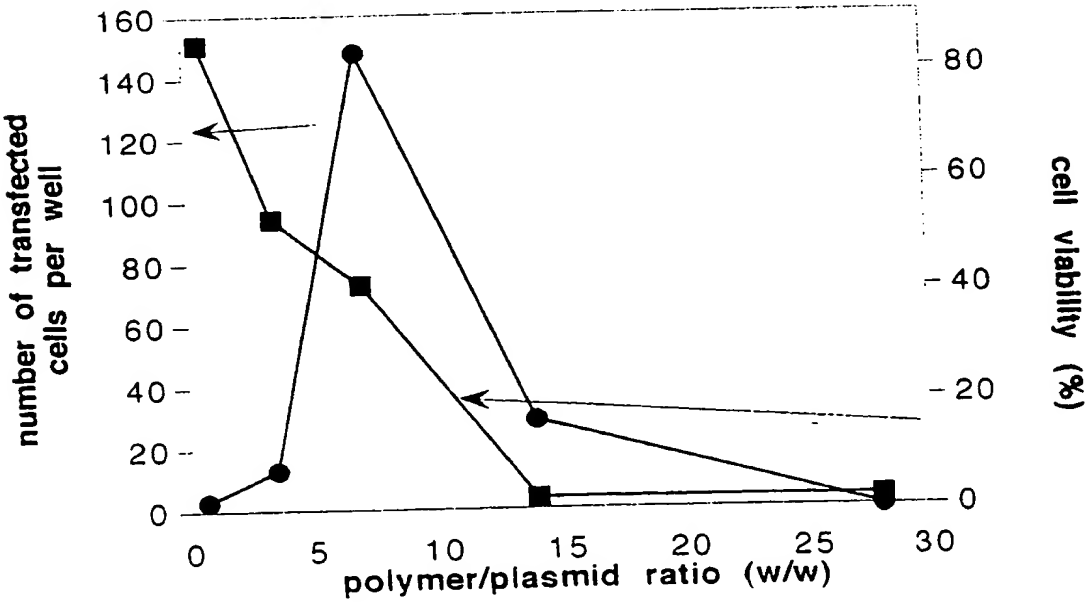


Figure 6:

Polymer: Mw = 600 kDa, degree of substitution 100 %, substituents: N,N-dimethylethanol amine and PEGME 2/1 mol/mol



INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/NL 96/00324

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/87 A61K47/48 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,95 32736 (THE PENN STATE RESEARCH FOUNDATION) 7 December 1995 see page 6 - page 8 see page 13, line 7 - line 20 see page 14, line 15 - line 30 see page 15, line 1 - line 26 see page 2 lines 2-7, line 30 see page 18 see claims 1,6,8,9,16,17,21 ---	1,2,4, 8-10
Y	DATABASE WPI Section Ch, Week 9204 Derwent Publications Ltd., London, GB; Class B04, AN 92-028935 XP002014068 & JP,A,03 275 733 (TEIJIN KK) , 6 December 1991 see abstract --- -/--	1-6,9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 November 1996	15. 11. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Gac, G

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCONJUGATE CHEMISTRY, vol. 6, no. 1, February 1995, pages 7-20, XP000494803 KABANOV ET AL.: "DNA complexes with polycations for the delivery of genetic material into cells" see the whole document	1-6,9
A	EP,A,0 425 268 (TEIJIN LIMITED) 2 May 1991 see column 3, line 21 - line 35 see column 4, line 19 - line 22 see column 6 - column 8 see column 10, line 22 - line 25 see column 14, line 33	1-10
A	IL FARMACO, vol. 49, no. 1, January 1994, pages 69-74, XP000604659 CALICETI ET AL.: "Aminoacid esters and imidazole derivatives of polyphosphazenes : influence on release of drugs, degradability and swelling" see page 70 see page 73	1,2,9,10
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A	MACROMOL. CHEM. PHYSICS, vol. 193, no. 6, 1992, pages 1261-1271, XP000603336 CROMMEN ET AL.: "Synthesis and properties of water-soluble polyphosphazenes with aminoalcoholic functions" see the whole document	1-3,9
A	WO,A,95 02415 (VIRUS RESEARCH INSTITUTE) 26 January 1995 see page 7 see page 10, last paragraph see page 14 - page 15	1,3,4,9, 10
A	POLYMER ADVANCED TECHNOLOGY, vol. 3, no. 6, 1992, pages 279-292, XP000324934 DOMB ET AL.: "Degradable polymers for site-specific drug delivery" see page 279 - page 280 see page 283 see page 288 - page 289	5,8-10

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/NL 96/00324

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANTI-CANCER DRUGS, no. 3, 1903 - June 1992, pages 175-209, XP000430668 DUNCAN : "Drug-polymer conjugates : potential for improved chemotherapy" see page 182 see page 189	4-6
A	PROC. NATL ACAD. SCI., vol. 89, September 1992, pages 7934-7938, XP000371760 WAGNER ET AL.: "Influenzae virus haemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by tarnsferrin-polylysine-DNA complexes : Toward a synthetic virus-like gene-transfer vehicle" see the whole document	7
A	WO,A,92 05778 (MASSACHUSSETTS INSTITUTE TECHNOLOGY) 16 April 1992 see the whole document	1-4,8-10
A	WO,A,95 08320 (VIRUS RESEARCH INSTITUTE) 30 March 1995 see page 8, line 36 see page 9, line 19 see page 10, line 33 - line 34 see claims 4,11	1-4,8-10

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/NL 96/00324

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 10
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 10 is (at least partially) directed to a method of treatment of (diagnostic method practised on) the human/animal body (Art. 17.2.a.i) and Rule 39.2.iv)PCT, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 96/00324

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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